

RESEARCH ARTICLE

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## Non-systemic fungal endophytes in *Carex brevicollis* may influence the toxicity of the sedge to livestock

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### Abstract

The sedge *Carex brevicollis* is a common component of semi-natural grasslands and forests in temperate mountains of Central and Southern Europe. The consumption of this species causes a severe toxicity to livestock, associated to high plant concentrations of the  $\beta$ -carbolic alkaloid brevicolline. This research was started to ascertain the origin of this toxicity. An exploratory survey of alkaloid content in plants growing in contrasting habitats (grasslands/forests) did not contribute to find a pattern of the variable contents of brevicolline in plants, and led us to address other possibilities, such as a potential role of fungal endophytism. Systemic, vertically-transmitted endophytes producers of herbivore-deterrent alkaloids are known to infect many known forage grasses. We did not detect systemic endophytes in *C. brevicollis*, but the sedge harboured a rich community of non-systemic fungi. To test experimentally whether non-systemic endophytes influenced the synthesis of the alkaloid, 24 plants were submitted to a fungicide treatment to remove the fungal assemblage, and the offspring ramets were analysed for alkaloid content. Brevicolline was the major  $\beta$ -carbolic alkaloid detected, and the contents were at least five times lower in the new ramets that developed from fungicide-treated plants than in the untreated plants. This result, although not conclusive about the primary source of the alkaloid (a plant or a fungal product) indicates that fungal endophytes may affect the contents of the toxic brevicolline in this sedge.

**Additional key words:** livestock toxicity; alkaloid; brevicolline; fungal endophyte; plant-endophyte interaction.

### Introduction

This research was initiated to elucidate the origin of a livestock toxicity caused by the sedge *Carex brevicollis* (DC) (Fam. *Cyperaceae*). This perennial plant is a common component of environmentally valuable grasslands and forests in temperate mountains of Central and Southern Europe. *C. brevicollis* is highly toxic to mammals, causing abortions in pregnant cows, ewes, and mares ingesting it, which results in severe economic damage to farmers (Ruiz de los Mozos *et al.*, 2008). Chemical analyses have reported a high content of  $\beta$ -carbolic alkaloids (up to 2% dry matter), mainly

brevicolline and to a lesser extent brevicarine, in the stems, leaves and inflorescences of this species (Sharipov *et al.*, 1975; Lazurjevski & Terentjeva, 1976; Busqué *et al.*, 2010). Brevicolline is known to enhance uterine contractions in pregnant mammals, producing an intense oxytocic effect (Yasnetso & Sizov, 1972). In addition, it has shown a strong antimicrobial activity against some bacteria and fungi in laboratory studies (Towers & Abramowski, 1983; Cao *et al.*, 2007).

The genus *Carex* encompasses about 1,800 species, many of them forage plants. Unlike other plant genera containing species exhibiting highly poisonous substances (*e.g.* *Ranunculus*, *Euphorbia*, *Lilium*, *Solanum*),

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Abbreviations used: EMBL (European Molecular Biology Laboratory); GC-MS (Gas Chromatography Mass Spectrometry); ITS (Internal Transcribed Spacer); PAR (Photosynthetically Active Radiation); PDA (Potato Dextrose Agar).

the existence of toxic alkaloids has not been documented in any other species of *Carex*, although some secondary chemicals such as proteinase inhibitors and stilbene derivatives have been described in some sedges as an induced response to grazing (Brathen *et al.*, 2004). These previous results and the nature of this particular toxicity, unique to this species, led us to suspect that the synthesis of alkaloids in *C. brevicollis* might respond to a mechanism of induction activated by a particular exogenous factor.

In the area of study this plant occurs in two well-differentiated habitats, forests and grasslands, both differing dramatically in light intensity and mammalian grazing pressure exerted on the sedge. These two factors are common inducers of chemical defences in angiosperms (Downum, 1992; Chen, 2008). Light has been shown to mediate the synthesis and activation of  $\beta$ -carboline, a set of phytochemicals derived from tryptophan, which includes brevicolline (Downum, 1992). Regarding herbivory, the induction of toxic compounds has been much more studied in plant-insect systems (Kessler & Baldwin, 2002; Castells *et al.*, 2005; Chen, 2008; Kaplan *et al.*, 2008) than in plant-mammal systems (Huntzinger *et al.*, 2004; Zinn *et al.*, 2007), although the occurrence of mechanisms of cross-resistance (defences induced by a particular herbivore being effective against other herbivores that consume the same plant) are assumed (Kessler & Baldwin, 2004). In plant-mammal systems, research has particularly addressed the loss of plant digestibility caused by the synthesis of defences against grazing/browsing (such as phenolic or silica-based compounds, Massey *et al.*, 2007), or it has focused on animals rather than plants, analysing the mechanisms developed by mammals to avoid or tolerate toxicity (Torregrossa & Dearing, 2010).

Plant-associated fungi are another factor linked to the synthesis of antiherbivore compounds in plants. Some of the best known examples of livestock poisoning by alkaloids involve endophytes, fungi that can asymptotically infect plants. The symbiosis between systemic endophytes of the genera *Epichloë* and *Neotyphodium* and their grass hosts has been well described in the past decades. Grasses infected by these endophytes defend from herbivory through the toxic alkaloids produced by the fungi (Clay & Schardl, 2002; Rodríguez *et al.*, 2009). Similarly, different species of the sedge *Cyperus* spp. have shown antiherbivore activity and increased growth and survival when infected by systemic *Ba-*

*lansia* endophytes (Clay *et al.*, 1985; Stovall & Clay, 1988).

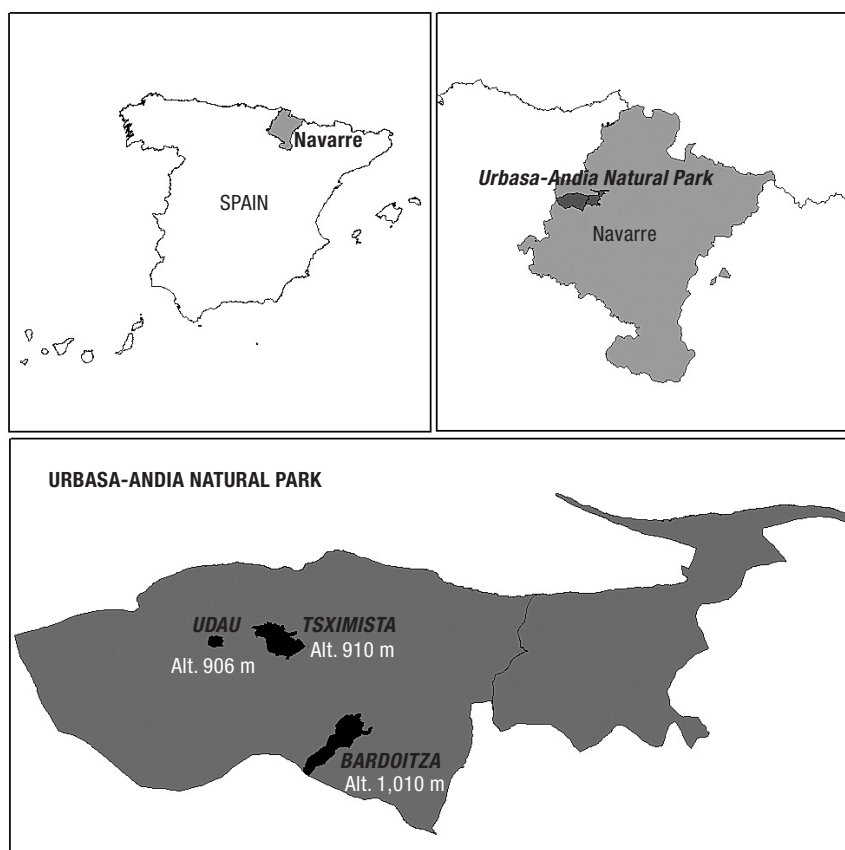
Unlike the well-known *Epichloë/Neotyphodium* species, most other fungal endophytes are not capable of systemic colonization of plant organs, or seed transmission (Sánchez Márquez *et al.*, 2012). These non-systemic endophytes are extremely diverse taxonomically, and have been found in all plant taxa analysed, including sedges (Ruotsalainen *et al.*, 2002; Rodríguez *et al.*, 2009; Loro *et al.*, 2012). The ecological functions attributed to non-systemic endophytes are very diverse, and unknown for most species (Saikkonen *et al.*, 1998; Rodríguez & Redman, 2008). Till date, few studies have addressed in particular the role of non-systemic endophytes in plant defensive mechanisms. The endophytic fungus *Undifilum oxytropis*, which infects several *Astragalus* and *Oxytropis* species, has been shown to produce the toxic alkaloid swainsonine (Cook *et al.*, 2009; Yang *et al.*, 2012) and some symbiotic epiphytic fungi living on plant surfaces have also been linked to plant toxicity caused by ergoline alkaloids in *Ipomea* species (Markert *et al.*, 2008).

Because of the above background, we first investigated a potential influence of habitat in the production of the alkaloid brevicolline in *C. brevicollis* plants. Contents of brevicolline in leaves were high, variable among individuals, and did not exhibit the patterns expected between habitats. Therefore, we addressed the study of a potential involvement of fungal endophytes in the synthesis of this powerful alkaloid.

## Methods

### Study site and plant sampling

This research was done in a mountainous rangeland area, Urbasa (Navarra, Spain), located south of the Western Pyrenees (950 m a.s.l., Fig. 1). The site, included in the Urbasa-Andia Natural Park, receives a temperate climatic influence (mean temperature = 8.4°C; rainfall = 1,275 mm yr<sup>-1</sup>) and is characterized by a karstic landscape covered by 11,400 ha of grasslands, heathlands, and beech (*Fagus sylvatica*) forests enclosed in the European Natura 2000 network. This area has been grazed extensively by livestock since the Neolithic period, and nowadays supports the pressure of more than 13,500 sheep, 2,500 cows and 750 horses from May to October each year. Intoxications caused by the consumption of *C. brevicollis* occur every year



**Figure 1.** Study area and locations within the Urbasa-Andia Natural Park in Navarra, Spain.

in Urbasa, although several farmers have implemented livestock management measures to reduce the risk (Ruiz de los Mozos *et al.*, 2008). *C. brevicollis* grows almost everywhere in the area, both in closed and open habitats. The former, constituted by beech forests, occupy about 73% of the surface and holds a poor understory due to high tree density and canopy development. The open habitats are constituted by a mosaic of grasslands and heathlands that are intensively grazed during the plant growth season.

Compared to open areas, within the forest understory light is dramatically attenuated. In a mid-day measurement before the autumn leaf fall, we observed photosynthetically active radiation (PAR) photon flux attenuations 20 times greater in the forest understory. Regarding grazing, it is almost absent in the forest, whereas open areas support a high livestock pressure during the grazing period, with an average of 2 cows  $\text{ha}^{-1}$ . By means of studying plants from such contrasting ecosystems, we intended to gain insight into the nature of the chemical defence of *C. brevicollis*.

Samplings were done at two different locations (Fig. 1), Udaú ( $42^{\circ}50'N$   $2^{\circ}8'W$ ), and Bardoitza ( $42^{\circ}48'N$   $2^{\circ}4'W$ ), where mosaics of grassland and beech forest habitats occurred. In autumn, at the end of the grazing period, 40 plants were collected in total, 20 per location, of which 10 grew in grasslands and 10 in the understory of adjacent beech forests. At each location, we selected grassland and forest habitats less than 30 m apart, that shared similar physiographical traits (aspect, topography, slope and substrate), but that differed radically in vegetation type, intercepted light and grazing intensity. Most *C. brevicollis* plants in grasslands were partially defoliated by grazing, whereas forest plants displayed no defoliation signs.

### Isolation and identification of fungal endophytes

A survey of fungal endophytes associated to leaves of *C. brevicollis* was made with the 40 plants collected

in grassland and forest habitats in Udaú and Bardoitza. To estimate the amount of endophytic colonisation of the plants, we diagnosed the presence of endophytes in samples of 26 leaf fragments per plant. Samples were obtained cutting transversally several asymptomatic leaves from each plant in fragments of about 5 mm of length. The fragments were superficially disinfected by immersion in a solution of 20% domestic bleach (1% active chlorine) for 10 minutes, rinsed in sterile water, and placed in two Petri plates containing potato dextrose agar (PDA) with 200 mg L<sup>-1</sup> chloramphenicol. The effectiveness of the surface disinfection method was tested with a sample of several leaf fragments using the tissue print method described by Schulz *et al.* (1998). The plates containing the leaf samples were incubated in the dark at room temperature (22-26°C), and checked daily for the presence of fungal mycelium emerging from leaf fragments. When this was observed, a sample of the mycelium was transferred to another PDA plate to obtain a culture for later identification, and the infected leaf fragment was withdrawn from the Petri plate, excising the agar around it to avoid fungal colony growth. Three weeks after plating the leaf fragments, we recorded the total number of endophyte-infected leaf fragments from each plant. These data were analysed to compare the amount of endophytic colonisation per plant at both locations and types of habitats.

For the identification of endophytes, the fungal isolates obtained from leaf fragments were grouped into morphotypes, according to macroscopic characteristics such as colony appearance and colour (Sánchez Márquez *et al.*, 2007). Afterwards, one or more isolates representative of each morphotype were identified using microscopic and molecular characters. Only the morphotypes consisting of more than one isolate were identified this way. The molecular character used was the nucleotide sequence of the ITS1-5.8S rRNA-ITS2 region, which was obtained after amplifying this region by PCR (Sánchez Márquez *et al.*, 2007). Nucleotide sequences were used to find similar matches in the European Molecular Biology Laboratory (EMBL) nucleotide database. To assign taxa to the sequences, genus and species of the closest database match were accepted when the sequence similarity of the *Carex* endophyte and the database match was greater than 98%, only the genus was accepted when the similarity was between 97.9 and 95%. When similarities were lower than 95% the isolates were considered as unidentified. Such criteria for ITS-based identification of

fungi has been found appropriate in other endophyte surveys (Sánchez Márquez *et al.*, 2007).

### Brevicolline alkaloid determination

In the laboratory we separated several ramets from each of the 40 plants sampled, and used their leaves for alkaloid extraction. Several extraction procedures were tested before choosing the one proposed by Zayed & Wink (2005). One gram of ground plant tissue was treated with 25 mL 1 M HCl overnight at room temperature. Then, the solution was filtered and alkalised to pH 12 with 6 M NaOH. The alkaloids were extracted by washing three times with 30 mL dichloromethane, and filtered through MgSO<sub>4</sub> to eliminate water traces. Collected samples were *vacuum* evaporated and kept at 4°C. Previous to GC-MS, alkaloids were separated in a *Factor Four* column (VF-5ms, 30 m × 0.25 mm, DF = 0.25 µm). *C. brevicollis* alkaloids are unusual and particular to this plant, so commercial standards are not available. For this reason, nicotine was used for the GC-MS analysis, since it is a common alkaloid from which brevicolline and similar β-carbolic structures can be obtained (Wagner & Comins, 2006). Brevicolline was identified and estimated quantitatively using two different complementary GC-MS techniques, electronic impact ionization, that gives the standard fragmentation of the molecules, and chemical ionization, which determines its molecular weight and estimates the number of molecules present.

### Experimental fungicide treatment of *Carex brevicollis*

To check whether the presence of fungal endophytes affected the alkaloid content in host plants, we designed an experiment consisting of eliminating the endophytic mycobiota of field sampled plants using a systemic fungicide, and then comparing the alkaloid content of the new ramets produced by these plants with that of the corresponding ramets of untreated plants and of the mother plants sampled in the field. Twelve apparently healthy plants of *C. brevicollis* collected in three different grasslands in Urbasa (Tximista, Udaú and Bardoitza) were planted in pots containing an organic soil mixture, and maintained for several weeks in the greenhouse with a constant watering regime. After this period, we separated three ramets from each mother plant. The mother plants were harvested and stored at -20°C

for further analyses of alkaloid contents while the new ramets were transplanted to new pots in the greenhouse. One ramet was kept as a control and the remaining two were treated with the systemic fungicide propiconazole (Oid-Zol®, Tragusa), which inhibits ergosterol synthesis. Ergosterol is critical for the formation of fungal cell walls, and its absence prevents fungal growth and further invasion of host tissues. The treatment consisted of three doses of 800 µg of propiconazole per plant spaced 10 days. The first and third doses were applied to the soil, due to the upward systemic movement of propiconazole from the roots to the foliage, and the second to the leaves (Zabalgogazcoa *et al.*, 2006). Treated and untreated plants were then allowed to grow for 50 days in the greenhouse until new ramets developed. The new ramets were collected and stored at -20°C until the alkaloid content was determined with the same analytical protocol previously described. The reason for analysing new ramets produced by these plants was to avoid possible fungicide effects upon alkaloid synthesis. As a whole, 48 samples were collected for alkaloid analyses (12 mother plants, 24 ramets developed from fungicide treated plants, and 12 ramets developed from untreated control plants).

### Statistical analyses

Prior to the use of parametric statistics, data were checked for normality and homogeneity of covariances, and transformed when necessary in logarithmic variables. We performed an ANOVA with habitat (grassland/forest understory) as a fixed factor and location (Udau, Bardoitza) as a blocking factor, in order to discern whether contents of brevicolline differed significantly between habitats. For the study of endophytic colonisation, we performed multifactorial ANOVAs where habitat was a fixed factor, location a blocking factor and the response variables were the number of endophyte-infected fragments per plant and the number of *Biscogniauxia nummularia* isolates.

Data from the fungicide experiment were analysed using a linear mixed model with the following factors: treatment (with three levels: mother, fumigated and unfumigated plants), origin of the plants (12 original plants) and grassland (three different grasslands, Bardoitza, Tximista and Udau). Treatment was considered a fixed factor and origin of the plant nested within grassland a random factor. In the case of fumigated ramets (24), the data used was the mean obtained from

the two fumigated ramets with the same ancestor (12). In order to choose the structure of the covariance, likelihood ratio tests were performed to compare different models. The most parsimonious model was fitted with a scaled identity covariance structure in which the elements are not correlated and have a constant variance. All statistical procedures were carried out using the IBM SPSS statistics package.

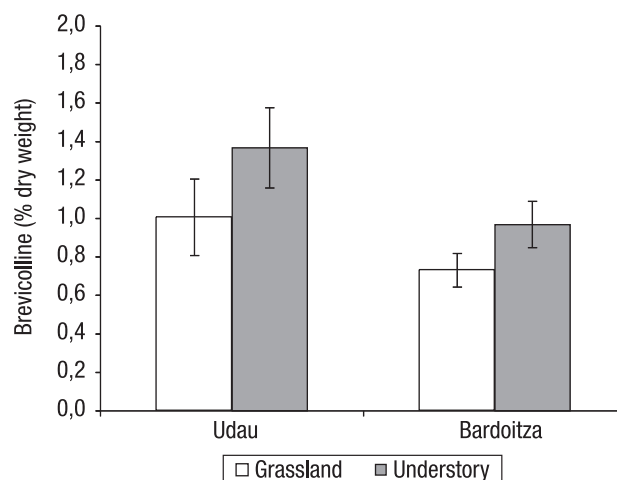
## Results

### Alkaloid contents in natural populations of *Carex brevicollis*

The combined techniques used in the GC-MS analysis allowed the identification of a group of similar  $\beta$ -carbolic organic structures, whose major component corresponded to the alkaloid brevicolline. All plants analysed contained brevicolline in highly variable amounts, ranging from 0.224 to 2.863 g kg<sup>-1</sup> dry matter. Brevicolline concentrations tended to be lower, although the difference was not significant, in plants growing in grasslands than in those from the forest understory ( $F_{1,36} = 3.409$ ;  $p = 0.073$ ) (Fig. 2).

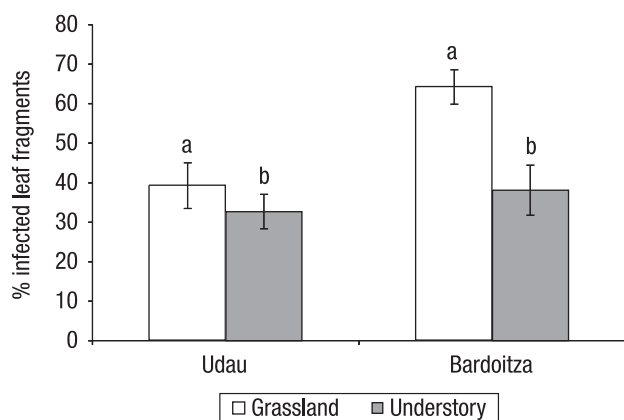
### Endophytic colonisation of leaves of *Carex brevicollis*

The amount of endophytic colonisation of leaves was estimated as the percentage of infected leaf frag-



**Figure 2.** Contents of brevicolline (average  $\pm$  standard error) in the leaves of plants growing in grassland and in forest understory habitats at the two locations surveyed. Contents of the alkaloid show a tendency to be significantly different between habitats ( $F_{1,36} = 3.409$ ;  $p = 0.073$ ).





**Figure 3.** Amount of endophytic colonisation (mean  $\pm$  standard error) in plants of grassland and forest understory habitats ( $F_{1,36} = 7.843$ ;  $p = 0.008$ ). The estimations are based on the average number of endophyte infected leaf fragments per plant at each location and habitat. Different letters indicate the occurrence of significant differences (LSD;  $p < 0.01$ ).

ments per plant. In Udaú an average of 36% of the fragments of each plant were colonised by endophytes, and in Bardoitza 51.2% of the fragments. At both locations the amount of endophytic colonisation was greater in plants from grasslands (51.6%) than in those from

nearby forests (35.3%) (Fig. 3), and this habitat effect was statistically significant ( $F_{1,36} = 7.837$ ;  $p = 0.008$ ).

### Endophyte identification

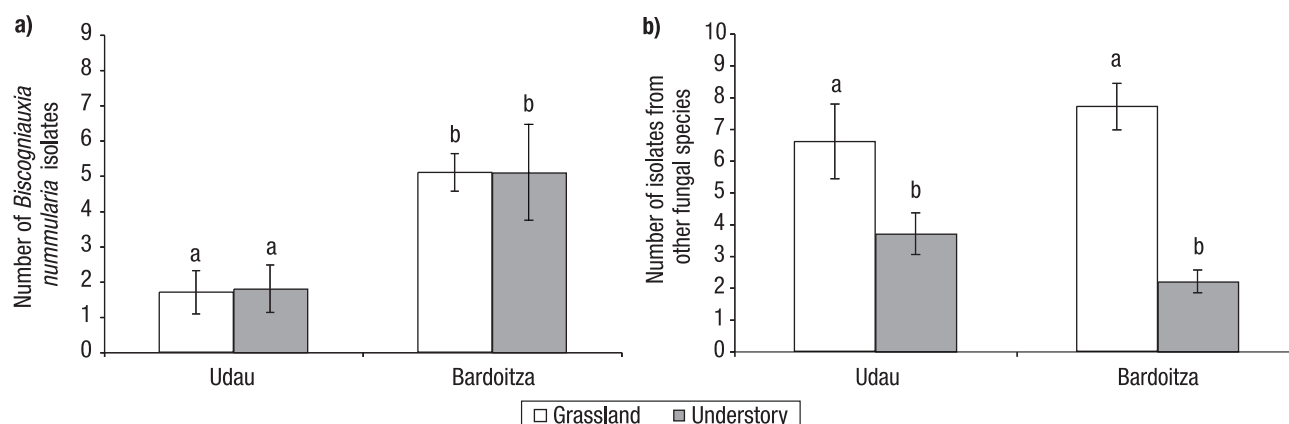
The leaves of *C. brevicollis* supported a rich and abundant fungal assemblage. From the 40 plants analysed, 347 fungal isolates were obtained and grouped into 103 different morphotypes. Nineteen morphotypes contained more than one isolate and 263 isolates were classified into these plural morphotypes. The remaining 84 morphotypes were unique, each consisting of a single isolate. Only the plural morphotypes were identified using nucleotide sequences, and in this group morphological characters were also used for identification of cultures that sporulated. With this information the 19 plural morphotypes could be regrouped into 14 taxa, indicating that calculations based on morphotypes overestimated the actual number of taxa (Table 1). All identified filamentous fungi, including unknown taxa, were ascomycetes, as deduced from their placement in a phylogenetic tree of ITS sequences.

The most abundant endophytic taxon was *Biscogniauxia nummularia*, 140 isolates of this species

**Table 1.** Fungal endophytes identified in the leaves of *Carex brevicollis*. Ten plants were sampled and analysed at each habitat and location. The number of isolates obtained and the proportion (in parenthesis) of these ten plants that were infected by each fungus are shown for grasslands (G) and beech forests (F) in the two sampling locations, Udaú (U) and Bardoitza (B). Only endophytic species represented by more than one isolate are listed

Endophyte identity	Location and habitat				Total
	UG	UF	BG	BF	
<i>Biscogniauxia nummularia</i>	17 (0.6)	17 (0.7)	52 (1.0)	54 (0.9)	140
Unknown ascomycete 1	11 (0.1)	1 (0.1)	2 (0.2)	14 (0.9)	28
<i>Colletotrichum</i> sp.	5 (0.4)	2 (0.1)	14 (0.6)	—	21
Unidentified yeasts	6 (0.3)	7 (0.4)	3 (0.2)	—	16
<i>Hypoxyton fragiforme</i>	1 (0.1)	1 (0.1)	4 (0.4)	5 (0.2)	11
Unknown ascomycete 2	6 (0.1)	—	—	5 (0.1)	11
<i>Alternaria</i> spp.	—	—	9 (0.3)	—	9
<i>Cladosporium</i> sp.	3 (0.2)	2 (0.2)	1 (0.1)	—	6
<i>Neofabraea alba</i>	2 (0.1)	—	3 (0.3)	—	5
<i>Penicillium</i> sp.	1 (0.1)	1 (0.1)	1 (0.1)	2 (0.2)	5
<i>Gibberella</i> sp.	4	—	—	—	4
Unknown ascomycete 3	1 (0.1)	—	2 (0.1)	—	3
Unknown ascomycete 4	—	—	—	2 (0.1)	2
Unknown ascomycete 5	—	2 (0.1)	—	—	2
Isolates of other taxa <sup>1</sup>	30	12	29	13	84
Total number of isolates	87	45	120	95	347

<sup>1</sup> Taxa classified as unique morphotypes were not identified.



**Figure 4.** Mean number ( $\pm$  standard error) of *Biscogniauxia nummularia* isolates (a) and isolates from other fungal species (b) obtained per plant at the two locations and habitats. Different letters (LSD;  $p < 0.001$ ) indicate differences between habitats (grassland/forest understory).

were obtained, and 80% of the plants were infected by it. The number of isolates of *B. nummularia* was very similar in grassland and forest habitats (Fig. 4a), but levels of infection were much higher in Bardoitza than in Udau (Table 1). An unknown ascomycete was the second most abundant taxon. This species and five others could not be identified because their cultures were sterile in PDA, and their nucleotide sequences were less than 95% similar to any identified accession from the EMBL nucleotide sequence database (Table 1). Contrary to *B. nummularia*, which displayed a similar number of isolates in closed and open habitats (Fig. 4a), the number of isolates of the other fungal species identified was significantly greater in grasslands than in nearby forests (Fig. 4b;  $F_{1,36} = 28.580$ ;  $p < 0.0001$ ).

After the above results were obtained, a second set of six plants of *C. brevicollis* was sampled in Bardoitza grassland and processed for endophyte isolation, with the purpose of obtaining more isolates of *B. nummularia*. This fungus is easy to distinguish from other endophytes because of the brown coloration of its colonies. After the field sampling, *B. nummularia* was well isolated from two of the six plants, but six months later, neither *B. nummularia* nor any other fungi was isolated from the new ramets that had developed in the greenhouse.

### Alkaloid contents in fungicide-treated ramets of *Carex brevicollis*

Beta carbolic alkaloids were detected in extracts of all the new ramets produced by *C. brevicollis* plants,

in those produced by fungicide-treated plants as well as in those derived from the untreated control ramets. The toxic brevicolline was the major alkaloid detected by GC-MS analysis, it was present in all the individuals and represented  $96.2\% \pm 2.2$  (mean  $\pm$  standard error) of the  $\beta$ -carbolic structures present. The concentrations of  $\beta$ -carbolic alkaloids were more than five-times lower in ramets developed from fungicide-treated plants than in those that grew from non-treated plants (Table 2; Fig. 5). Among non-treated plants, concentrations were significantly higher in mother plants sampled in the field than in their offspring ramets produced in the greenhouse.

## Discussion

*C. brevicollis* is the only toxic species of a genus of forage plants, and is able to grow successfully in plant communities with an extended grazing history. In this survey, brevicolline contents displayed high variability among plants, and did not differ significantly between open and closed habitats. The alkaloid, contrary to our expectations, tended to be higher in plants growing in the forest understory, where grazing is unusual and light scant. Although our work was not designed to specifically test the effect of grazing on brevicolline synthesis, these observations suggest that grazing might not be a first order factor affecting brevicolline content in plants. Two main abiotic parameters, growing degree days and altitude, were not found to be related to the brevicolline content of leaves of *C. brevicollis* in a survey where variable concentrations

**Table 2.** Estimates of the fixed and random effects from the experiment of the fungicide treatment. The dependent variable is the number of molecules of  $\beta$ -carbolic alkaloid. Fixed factor: treatment with three levels, mother, non-fumigated and fumigated plants. Random effect: origin of the plants (12 original plants) nested within grasslands (Udau, Bardoitza and Tximista). Type of covariance of the random effect: scale identity

Fixed effects	Numerator DF <sup>1</sup>	Denominator DF	F	Significance
Intercept	1	14.555	50.904	0.000
Treatment	2	32.720	12.028	0.000
Random effects	Estimate	Standard Error	Wald Z	Significance
Parameter				
Residual	7.68·10 <sup>11</sup>	1.94·10 <sup>11</sup>	3.962	0.000
Plant origin (grassland)	9.39·10 <sup>9</sup>	9.91·10 <sup>10</sup>	0.095	0.925

<sup>1</sup> DF = degrees of freedom.

of brevicolline also occurred among plant individuals (Busqué *et al.*, 2010).

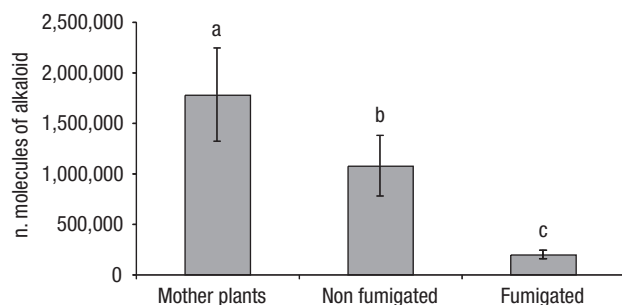
The endophyte survey revealed the existence of a rich and diverse fungal community in the leaves of *C. brevicollis*, dominated by 14 taxa that comprised 75.7% of all isolates obtained. The amount of endophytic colonisation was higher in plants from grasslands. This could be due to the greater amount of tissue wounds caused by grazing, which might facilitate the entry of horizontally transmitted endophytes, or perhaps to the tendency of higher contents of brevicolline in the forest sedges, which may exert a control on microbial plant populations.

The dominant endophytic taxon was *Biscogniauxia nummularia*, found at both locations and habitats, and in 80% of the plants analysed. Despite the high prevalence of this fungus as an endophyte in wild populations of *C. brevicollis*, we did not recover it from new

ramets produced in the greenhouse by infected mother plants. This led us to suspect that, in spite of its abundance, the colonisation of *C. brevicollis* by *B. nummularia* was of a non-systemic type (Sánchez-Márquez *et al.*, 2012). It is interesting that *B. nummularia* is known for being an endophyte and a pathogen in *Fagus sylvatica* (Hendry *et al.*, 2002), which is the dominant tree species in the area of study.

In the last decades, several studies have shown that non-systemic endophytes are ubiquitous in plant species and compose an extremely diverse phylogenetic group. The roles and functions of these fungi in the host plants are mostly unknown and are the focus of interesting studies (Rodriguez & Redman, 2008; Zabalgogea, 2008). From an evolutionary perspective, horizontal transmission and high fungal diversity within the host are more consistent with antagonistic, rather than mutualistic interactions between the host and its symbiont. The according theory of balanced antagonism states that in plant-endophyte interactions there is a degree of virulence by the fungal partner, the host plant having to defend to maintain fungal invaders below a given threshold (Schulz & Boyle, 2005). However, other authors believe that the long evolutionary interaction and the pervasive occurrence of endophytism indicates that fungi might compensate the cost of heterotrophism by playing some positive functions in the plant, such as improved adaptation to biotic and abiotic stresses (Arnold & Lewis, 2005; Rodriguez & Redman, 2008; Zabalgogea, 2008; Saikkonen *et al.*, 2010; Yuan *et al.*, 2010).

The synthesis of toxic alkaloids by non-systemic endophytic fungi has been discovered in some plant



**Figure 5.**  $\beta$ -carbolic alkaloid contents in mother plants and in the new ramets developed from treated and non-treated individuals of *Carex brevicollis*. Compared to fumigated plants, alkaloid contents in mother plants and in untreated ramets increase  $\times 9$  and  $\times 5$ , respectively.



species in recent years (Cook *et al.*, 2009; Yang *et al.*, 2012). In these cases, a particular fungus with an endophytic lifestyle is able to produce *in planta* and *in vitro* a toxic alkaloid. In our experiment ramets derived from fungicide treated plants produced alkaloids, but in much lower quantities than ramets from non-treated plants. At first sight, these results suggest that the alkaloid is a plant product that can be induced by fungal endophytism. The synthesis of indole alkaloids, such as  $\beta$ -carbolines, elicited by fungal cell walls has been shown in plant cell cultures (Shanks *et al.*, 1998; Facchini, 2001; Zhao *et al.*, 2001; Bais *et al.*, 2003; Pauw *et al.*, 2004). Although a plant synthesis with a fungal regulation is plausible for brevicolline, more research is still needed since other explanations are possible. Different endophytic agents inhabiting plants, such as bacteria, may play a role in the synthesis of toxins (Zhang *et al.*, 2006). Besides, in some cases, endophytes might be incompletely removed by fungicides (Cheplick, 1997; Faeth & Sullivan, 2003), what opens up the possibility of a fungal synthesis of the toxin. It is presumable that the fungicide treatment removes non-systemic endophytes better than systemic ones but, on the other side, a re-infection is easier to occur in the former than in the latter. Further research is needed to test these ideas, and to analyse whether *B. nummularia*, the most abundant fungal endophyte, has a particular role on toxin regulation.

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